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Homoplasmy stability of transplastomic tobacco plants (*Nicotiana tabacum* CV. Xhanti) containing human tissue-type plasminogen activator (*K2S* form) gene in T_1 generation

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Abstract

The rates of recombinant protein in nuclear-transformed plants are often less than 1% of total soluble proteins. As the plant plastid is highly polyploidy, plastid transformation can lead to high-level production of recombinant protein. In addition, plastid transformation has several other advantages such as prevention of gene escape that has a high importance in molecular farming. Tissue-type plasminogen activator (*tPA*) is an important protein that is used to treating clots in cardiovascular diseases. Thus, production of *tPA* protein in plant system was considered. The *tPA* (*K2S* form) gene was transferred to tobacco chloroplast genomes. In this study, we analyzed expression and stability of *tPA* gene in transplastomic tobacco plants in T_1 generation. The presence of *tPA* gene in transplastomic plants was confirmed with specific primer by PCR analysis. Homoplasmy, gene expression, and protein assay were confirmed with southern blot, RT-PCR, western blotting and ELISA analysis. Results showed that the *tPA* gene in T_1 generation of transplastomic tobacco plants is stable and is expressed. In addition, the maximum amount of *tPA* protein was estimated up to 0.13% of total soluble protein.

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Introduction

The production of recombinant proteins in plants has several advantages than the other systems, based on animal cells, yeast, and bacteria, including very low production costs, very high production potential, and pathogen-free products (Ma *et al.*, 2003). Also plants are eukaryotic organisms, so they can perform post-translational modifications (folding, glycosylation, *etc*) similar to animal cells on target proteins (Scotti *et al.*, 2012). The target gene can be inserted in either nuclear or plastid genome to stable transformation, or be expressed transiently. In nuclear stable transformation, expression level generally is low, so often maximum amount of expression less than 1% of total soluble proteins (TSP) have been reported (Sheludko, 2008). However, plastid transformation can lead to high-level production of recombinant protein because the plants can be contained up to 10,000 copies (number of plastid genome) of target gene in a cell, also gene silencing and positional effects are absent in plastid transformation (Rigano *et al.*, 2009). In addition, there are several other advantages in plastid transformation including prevention of gene escape because of maternal inheritance of plastids (Obembe *et al.*, 2011), and increased stability of heterologous protein in stroma compared to cytoplasm (Rigano *et al.*, 2009).

Tissue-type plasminogen activator (*tPA*) is a serine protease enzyme that can activate plasminogen to its active form, plasmin. Plasmin lyses clots by breaking-down the fibrin in blood clots formed in the vessels. Therefore, *tPA* can be used in treating cardiovascular and cerebrovascular obstructions (Rouf *et al.*, 1996). For plasminogen activation, *tPA* should be placed adjacent to plasminogen on the fibrin in clots, which means that *tPA* can not activate circulating plasminogen in the blood (Baruah *et al.*, 2006).

The *tPA* is a single chain polypeptide that consisting of 527 amino acids whit 17 disulfide bonds between 34 cysteine residues. In addition, *tPA* is glycosylated and its molecular weight is approximately 70 kDa. Five domains have been identified in *tPA* including a fibrin-binding 'finger' domain, an epidermal growth factor, two disulfide looped 'kringle' domains, and a serine protease domain which is involved in proteolytic activity at carboxyl terminal of polypeptide chain (Rouf *et al.*, 1996).

Reteplase is non-glycosylated truncated form that contains two domains of native *tPA*. It is composed of 355 amino acids with 39 kDa molecular weight. In fact, this form of *tPA* comprises kringle-2 and serine protease domains. Reteplase has a longer half-life (14 min) and higher thrombolytic potency compared to native form. In addition, formation of fibrin-plasminogen-*tPA* complex is not necessary to accurate function of reteplase (Baruah *et al.*, 2006). The DNA sequence encoding reteplase protein so-called *K2S*.

The *tPA* protein is found in different tissues of human and animals. For natural production of *tPA*, tissues including melanoma, epithelial, fibroblasts, and endothelial are used through tissue culture technique and *tPA* is purified from these tissues. Besides, recombinant DNA technology can be used to produce *tPA* synthetically (Rouf *et al.*, 1996). So far, *tPA* protein has been produced in mouse L cells (Browne *et al.*, 1985), Bowes melanoma cell line (Dodd *et al.*, 1986), mammalian cell lines (Jalanko *et al.*, 1990), *E. coli* (Obukowicz *et al.*, 1990), Chinese Hamster ovary (CHO) cells (Fann *et al.*, 2000), and Leishmania (Soleymani *et al.*, 2006). The production of *tPA* in plant can be a beneficial way to commercial production of this protein.

Two reports exist about efforts to production of *tPA* in tobacco plant (Hahn *et al.*, 2009; Masoumiasl *et al.*, 2010). However, tobacco plant in these studies was nuclear-transformed and low production rates have been reported. Hence, to increase expression level of *tPA* gene in tobacco plant, it (*K2S* form of *tPA*) was inserted in pKCZ vector and after cloning was transferred to tobacco chloroplast using biolistic procedure (Abdoli-Nasab *et al.*, 2013). Stability of transformation in chloroplast-transformed plants is very important, because the resulting plants are cultivated in next generation to production of

recombinant protein. If the transformation is not stable in next generation, the chloroplast-transformed plants are not suitable to cultivation and transformed chloroplasts quickly replaced with wild-type chloroplast. Therefore, chloroplast-transformed plants must be homoplast and this homoplasmy be stable in next generation. Thus, in this study we investigated the expression and stability of *K2S* gene in T_0 transplastomic plants that provided before (Abdoli-Nasab *et al.*, 2013) in T_1 generation.

Materials and methods

Seed culture and PCR analysis

The seeds of transplastomic tobacco (*Nicotiana tabacum* Cv. Xhanti) plants harboring pKCZ*K2S* construct (Fig 1a) were grown in pots contained a homogeneous mixture of perlite and peat moss (1:3 ratio).

Total genomic DNA was isolated from transgenic and wild type plant leaves using CTAB protocol (Doyle, 1987; Doyle and Dickson, 1987; Cullings, 1992). To confirm the presence of *K2S* gene at chloroplast genome, PCR analysis was carried out using specific primers (*K2S*-F 5´- GGA AAC AGT GAC TGC TAC TTT GGG AAT GG -3´ and *K2S*-R 5´- TCA CGG TCG CAT GTT GTC ACG AAT CCA G -3´) designed from *K2S* sequence. 50 ng of genomic DNA was used as template and PCR reaction condition were as 1 cycle of 5 min at 94°C followed by 30 cycles at 94°C for 30 s, 57°C for 40 s, 72°C for 90 s and a final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis on 1% agarose gel.

Southern blot analysis

Homoplasmy was confirmed in transplastomic plants by Southern blot analysis. At first, probe was synthesized using primers designed based on flank regions at chloroplast genome (F-F 5´-ATG TGT AAT GAT TCC CCC ATT C -3´ and F-R 5´-CTT CTC TCC CAC TTC ACA CCT C -3´). Double strand DIG-labeled DNA probe was prepared using DIG DNA-labeling Kit (Roche). Size of amplified fragment to probe was 350 bp. The total DNA was digested with Hind III enzyme; the digested DNA was electrophoresis on 1% agarose gel at 20 V voltage for 16 h. The DNA was transferred from gel on nitrocellulose membrane using a traditional wet system. The blotting procedure was carried out as according to the manufacturer's protocol (DIG-DNA labeling and detection kit (Roche)). The expected size of restricted fragment with Hind III enzyme in non-transplastomic plant was 1 kb and fragment size in transplastomic plants and positive control (pKCZ*K2S* vector) was 2.8 kb (fig 1b).

RT-PCR analysis

The southern blot positive plants were used to RT-PCR analysis. The total RNA was isolated from fresh tobacco leaves using RNX-plus solution according to manufacturer's protocol (Cinnagen, Iran, Tehran). The cDNA was synthesized using a RevertAid first strand cDNA synthesis kit (Fermentas). Specific primers were designed according to *K2S* sequence (*K2S*-F 5´- GGA AAC AGT GAC TGC TAC TTT GGG AAT GG -3´ and RT-R 5´- TTG ATG CGA AAC TGA GGC TG- 3´). PCR condition were as 1 cycle of 5 min at 94°C followed by 30 cycle at 94°C for 30 s, 57°C for 40 s, 72°C for 40 s and a final extension at 72°C for 10 min. The RT-PCR products were analyzed by electrophoresis on 1% agarose gel.

Western blot analysis

Total soluble protein (TSP) was extracted from fresh leaves of tobacco plants (Hurkman and Tanaka, 1986). Extracted proteins were separated by electrophoresis on 12% sodium dodecyl sulphate polyacrylamide gel (Laemmli, 1970). Then, proteins were transferred to nitrocellulose membrane using semi-dry transfer system according to manufacturer's instructions (Bio-Rad, USA). The detection of *tPA* protein was performed using rabbit polyclonal antibody *tPA* as primary antibody (Abcam, USA) at 1:1000 dilution and goat anti-rabbit IgG-HRP antibody (Santa cruz, USA) as secondary antibody at 1:1000 dilution. The bands were detected with H2O2 as substrate and 3, 3´-diaminobenzidine (DAB) detection system.

ELISA analysis

In order to estimate the amount of *tPA* protein in transplastomic plants, at first total soluble protein was extracted from fresh leaves of tobacco plants, then ELISA analysis was performed according to instructions as described (Abcam). Type and dilution primary and secondary antibodies were similar to western bolt analysis. The H_2O_2 and TMB (3, 3´, 5, 5´-tetramethylbenzidine) were used as substrate of HRP enzyme. Optical density (OD) was read at 450 nm wavelength.

Results

Amplification of K2S gene

The T_1 generation plants were analyzed for presence of *K2S* gene by PCR analysis. In three of T_1 plants and positive control (pKCZ*K2S* vector), the expected 1,059 bp fragment was amplified. No amplification was observed in the wild type plant (Fig. 2).

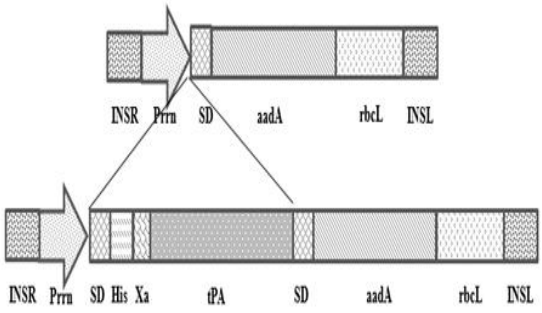

Fig. 1a. pKCZK2S vector for transformation of K2S gene in tobacco plastid. The K2S gene was iserted in pKCZ vector; the shindalgarno (SD) as ribosome binding site and six histidine amino acids (6-His) as tag to aim purification of tPA protein from cell extract was fused at upstream of K2S gene. In addition, the Xa protease restriction site was inserted between his-tag and coding sequence of K2S gene to elimination his-tag after purification of tPA protein. In this cassete both tPA and aadA genes were expressed as dicistronic.

Homoplasmy assessment in transplastomic plants

The three PCR positive plants were analyzed by southern blot analysis. In all three plants, the expected (2.8 kb) transplastomic band was revealed on membrane, while wild type band (1 kb) was not observable. Hence, these transplastomic plants were homoplast (Fig. 3).

Analysis of K2S transcripts by RT-PCR

In order to detection of *K2S* gene expression, RT-PCR was performed on three southern blot positive plants. The expected fragment (270 bp) was amplified in all three transplastomic plants. No amplification was observed in non-transplastomic plant and negetive control (Fig. 4). These results shown that *K2S* gene is expressed in transplastomic plants.

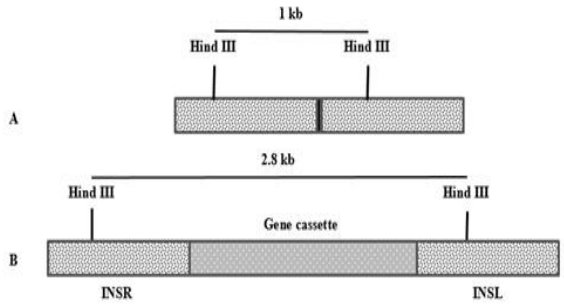

Fig. 1b. Size of restricted fragmens with Hin III enzyme in sothern blot analysis. Restriction of plastid genome of nontransplastomic plant was produed a 1 kb fragment (A) and size of restricted fragment in transplastomic plant was 2.8 kb (B). the probe was designed based on sequence between restriction site of Hind III enzyme in right flanke region (INSR) and gene cassette.

Analysis of tPA protein production in transplastomic plants

Western blot analysis was performed on transplastomic plants for detection of *tPA* protein production in transplastomic plants. The expected *tPA* protein band (39 kDa) was detected in all three transplastomic plants (Fig. 5). This showed that *K2S* transcripts were translated to *tPA* polypeptide successfully.

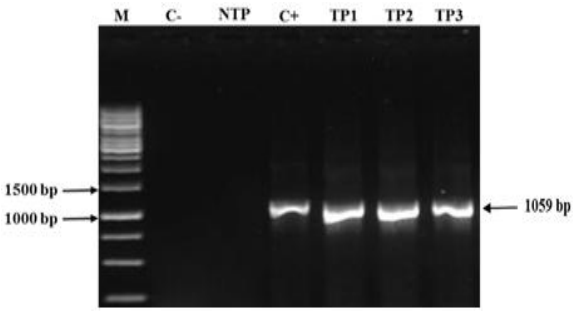

Fig. 2. PCR analysis of transplastomic plants with specific primers on the 1% agarose gel. M: 1kb DNA marker, C-: template-free reaction, NTP: nontransplastomic plant, C+: positive control (pKCZ

vector contain K2S gene), TP1-TP3: transplastomic plants.

Quantification of tPA protein in transplastomic plants

In order to quantification of *tPA* protein in transplastomic plants, ELISA analysis was performed. The highest amount of *tPA* protein was 0.13% of TSP (Fig. 6). The ELISA results showed that *tPA* protein was produced in all three transplastomic plants.

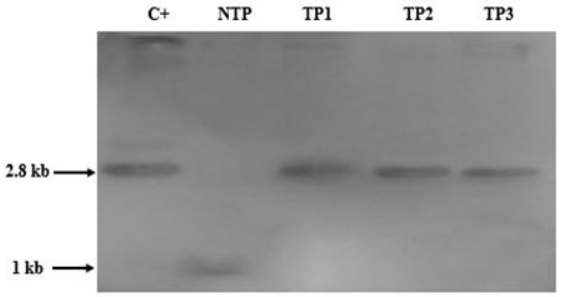

Fig. 3. Sothern blot analysis of transplastomic T1 Plants with Hind III enzyme. C+: positive control (pKCZ vector contain K2S gene), NTP: nontransplastomic plant, TP1-TP2: transplastomic plants.

Discusion

In this study, we analyzed T_1 generation of transplastomic tobacco plants in different levels (DNA, RNA, and protein). The PCR analysis using specific primers for *K2S* gene confirmed the presence of *K2S* gene in T_1 generation of plants and homoplasmy of three transplastomic plants was confirmed by southern blot analysis. In addition, the results showed the expression of *K2S* gene. Protein assay analysis confirmed the production of *tPA* protein in transplastomic plants. The maximum amount of 0.13% TSP for *tPA* protein was estimated in transplastomic plants. Different rates of recombinant protein production in transplastomic plants have been reported in various studies. In some studies rates below than 1% of TSP have been observed, for example production rates of 0.004 (Lee *et al.*, 2006), 0.1 (Zhou *et al.*, 2006), 0.2 (Li *et al.*, 2006), 0.3 (Wang *et al.*, 2008), 0.5 (Morgenfeld *et al.*, 2009; Sim *et al.*, 2009), 0.7 (Dreesen *et al.*, 2010), and 0.8 (Soria-Guerra *et al.*, 2009) of TSP have been reported (Cardi *et al.*, 2010). However, we can use some measures to enhance the production rates of recombinant protein in plants. Several factors are affecting recombinant protein production in plant system. Enhancement of transcription, translation, and protein stability can lead to high-level accumulation of recombinant protein.

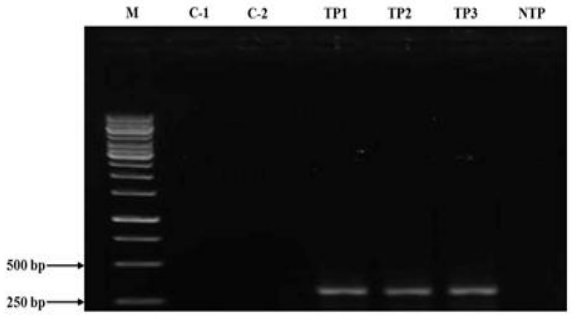

Fig. 4. RT-PCR analysis of transplastomic plants with specific primers on the 1% Agarose. M: 1kb DNA marker, C-1: negative control (templat free), C-2: negative control (RNA as a template), TP1-TP3: transplastomic plants, NTP: nontransplastomic plant.

Transcription

Use of a suitable promoter is one of the most important factors for increasing expression of transgene. In chloroplast transformation, promoters of genes with high-level expression in chloroplast organelle were commonly used. In this study, we used Prrn promoter that was widely applied in recombinant protein production in plants and high-level protein production has been reported using this promoter (Daniell *et al.*, 2009; Michelet *et al.*, 2011). Hence, selected promoter in our study is a suitable promoter to produce *tPA* protein in plastid transformation. Also, we can manipulate promoter and enhance transcription factor binding site in promoter sequence by modeling of many strong promoter to achieve a synthetic promoter (Egelkrout *et al.*, 2011).

The other factor affecting transcription is region of transgene insertion in plastid genome. In this study, *K2S* gene was inserted in site between trnN and trnR genes. In some investigations on plastid transformation, transgenes were often inserted in site

between trnA and trnI genes that is located in transcriptional active site in plastid genome (Maliga, 2012) and it can be contributed to increased expression of target gene. Both above mentioned sites are in inverted repeated (IR) region in plastid genome, hence transgene can be duplicated by the phenomenon of copy correction and be inserted into the other IR in plastid genome as well (Verma and Daniell, 2007).

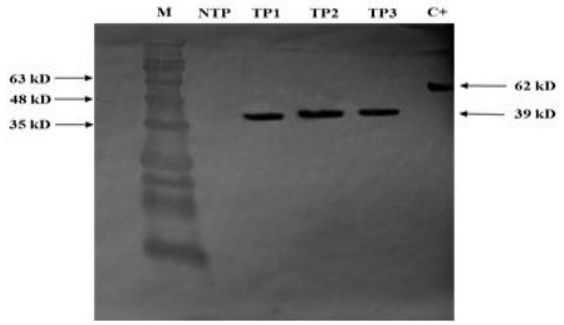

Fig. 5. Western blot analysis on transplastomic plants contain K2S gene. M: prestained protein marker, NTP: nontransplastomic plant, TP1-TP3: transplastomic plants, C+: positive control (alteplase).

Translation

After transcription of gene to mRNA, these gene transcripts need to be translated to protein. In many cases, there is not a direct correlation between mRNA levels and rates of produced protein (Millán *et al.*, 2003; Kim *et al.*, 2009). Also, it should be mentioned that in chloroplast, polyadenylation do not occur on mRNA transcripts and half-life of mRNA molecules might be shorter (Rigano *et al.*, 2009). Thus, consideration should be given to translation in production of recombinant protein in plant chloroplast.

In this study shinedelgarno sequence (SD) has been used upstream of coding region of *K2S* gene. This sequence is a prokaryotic ribosome-binding site (RBS). To enhance the translation conditions, use of 5´-UTRs that contain strong RBS elements can be beneficial. Also, these untranslated regions can improve mRNA stability in cell and cause mRNA transcripts to be subjected to translation by ribosome for longer time (Egelkrout *et al.*, 2011). On the other hand, in this study rbcL terminator has been used. This terminator in many investigations has been considered as an appropriate terminator (Oey *et al.*, 2008; Michelet *et al.*, 2011).

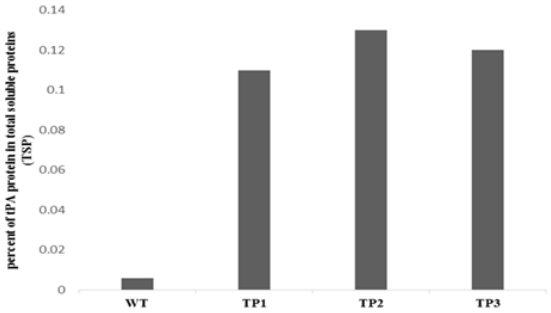

Fig. 6. Quantification of tPA protein in transplastomic plants. WT: nontransplastomic plant, TP1-TP3: transplastomic plants.

The ratio of codons used for a given amino acid differs between organisms and is correlated to the levels of tRNAs available for that amino acid. Thus, if target gene belongs to other organisms, use of codon optimization can be favorable for its expression in plants (Oey *et al.*, 2008; Zhou *et al.*, 2008). Thus, it is recommended to accommodate *K2S* gene codons to favorite codons of tobacco chloroplast to enhance rate of *tPA* protein production in transplastomic tobacco plants.

Protein stability

produced proteins in plants require to be accumulated and purified from plant tissues. Thus, produced protein in plant cell should be preserved from degradation by proteolytic processes. In nuclear transformation, produced protein is leaded to cell organelles or apoplast by subcellular targeting of recombinant protein. The production of recombinant protein in plant chloroplast has this potential advantage but this does not mean that there is no proteolytic processes and protease in chloroplast. various proteases and proteolytic activities in chloroplast were discussed before (Adam, 2007). Hence, protein degradation can be occurred in chloroplast and prevention of protein degradation is necessary to achieve higher levels of protein accumulation.

Half-life of truncated *tPA* (reteplase) is about 12-16 min (Baruah *et al.*, 2006), and *tPA* protein can be degraded immediately in cell, as we believe this is one of the most important reasons for low-level accumulation of *tPA* in tobacco plant. Hence, we should elevate half-life of *tPA* protein using suitable strategies. We can use of fusion proteins (tags) in N-terminal of protein to enhance stability of recombinant proteins, for example, thermo-stable proteins can be used, because there is an strong correlation between resistance to proteolysis and thermal stability, also peptide sequences without recognition site for proteases can be used as fusion to target recombinant protein to decrease the degradation of protein (Egelkrout *et al.*, 2011). However, it is obvious that use of any fusion protein should be well investigated to identify its effects on target protein.

Modification of amino acid residues in recognition sites of proteases in recombinant protein polypeptide sequence can increase the stability of protein (Amin *et al.*, 2004). Thus, protein engineering can be used as a beneficial tool to overcome post-translational degradation of recombinant protein. However, we are allowed to impose a modification that does not have an adverse impact on protein nature and function. These alterations can be performed when codon optimization is occurred on gene sequence.

As mentioned in introduction, plants have an acceptable potential as a host system to production of recombinant protein. In this study, we could approached to stable chloroplast transformed tobacco plants, this achievement is important because this plants can be cultured for years and recombinant protein to be produced. In addition, if mentioned alterations perform on our construct, high-level accumulation of *tPA* can be achieved in tobacco plant.

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